### Title

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# Inhibition of adenovirus serotype 14 infection by octadecyloxypropyl esters of 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl) nucleosides *in vitro*

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## ABSTRACT

The most common adenovirus serotypes 1, 3, 5 and 7 can cause respiratory infections, and serotypes 40 and 41 can cause gastrointestinal infections. Most adenovirus infections are usually mild or asymptomatic, but a re-emerged serotype, adenovirus 14, was reported to cause severe and fatal pneumonia in rare cases in people of all ages. Unfortunately, no antiviral compounds have yet been approved for the treatment of such adenovirus infections. Vaccines have been developed for only

two serotypes, 4 and 7, to prevent acute respiratory diseases (ARD) in military personnel. In this report, four nucleoside analog compounds, octadecyloxypropyl esters of 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl) derivatives of adenine (ODE-HPMPA), cytosine (ODE-HPMPC), guanine (ODE-HPMPG), and 2,6-diaminopurine (ODE-HPMP-DAP), were evaluated against adenovirus type 14 and several other adenoviruses *in vitro*. All the ODE-nucleoside analogues demonstrated selective antiviral activities in neutral red uptake and virus yield reduction assays. Time-of-addition assays revealed that the efficacy of each ODE-nucleoside analogue was present for up to 8 h following adenovirus infection of cells. Our data provide important insight into a serious of compounds that might be advantageous in treating adenovirus infections.

*Keywords:* A549 cells, adenovirus type 14, ODE-nucleoside analogues, 2',3'-dideoxycytidine

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Adenoviruses were first isolated in 1953 from human adenoids. Human adenovirus type 14 (AdV14) was first identified later in the 1950s. Based on the report from Centers for Disease Control and Prevention (CDC), as of September of 2007, outbreaks of AdV14 have been identified in New York, Oregon, Texas, Washington States with 10 deaths since May 2006 [1-4]. In February of 2007, an outbreak of acute respiratory infection with high fever was reported among trainees at Lackland Air Force Base (LAFB) in San Antonio, Texas [3, 4]. Among the patients tested, 268 were positive for adenovirus. Of 118 serotyped patients, 106 were identified as AdV14. 27 patients were hospitalized, one of whom, a 19 years old airman died of

AdV14 in Intensive Care Unit (ICU). In April of 2009, another airman died of this virus infection. On September 2008, another outbreak was caused by AdV14 in Prince of Wales Island, Alaska [5, 6]. O'Flanagan et al. reported the first 9 confirmed cases of AdV14 infection in Ireland from the October of 2009 to the July of 2010 [7]. All the isolates were distinct from the AdV14 reference strain from 1950s. A new AdV14 variant is termed the killer cold virus because of the high incidence of hospitalizations and deaths attributed to the viral strain [1]. These data suggested the emergence and spread of a new adenovirus 14 variant in the United States and Europe. Most recently, Zhang et al. presented the first genome of this new human adenovirus (referred to as – B14 strain), which had been isolated in Southern China [8]. Huang et al. reported an outbreak of febrile respiratory illness associated with human adenovirus type 14p1 in Gansu Province, China [9].

Adenoviruses are medium-sized (90-100 nm), non-enveloped icosahedral viruses composed of a nucleocapsid and a double-stranded linear DNA genome. There are 57 described serotypes in humans, which are responsible for 5-10% of upper respiratory infections in children, and many infections in adults as well. The most common adenovirus serotypes are 1, 3, 5 and 7 that can cause respiratory infections. Serotypes 40 and 41 can cause gastrointestinal infections. Most adenovirus infections are usually mild or asymptomatic. Viruses of the family *Adenoviridae* infect various species of vertebrates, including humans. AdV14 viruses are passed from person to person or picked up from items touched by the infected people and then initially invade the cells in the eye, nose, or mouth that subsequently allow further spread to other organs. AdV14 infections usually begin with cough, runny nose and fever as well as throat irritation. Some individuals have additional symptoms such as diarrhea, bronchitis, eye infections, bladder infection, rash, high fevers, pneumonia, and

shortness of breath. The vaccines have been developed for only two serotypes, 4 (AdV4) and 7 (AdV7), to prevent acute respiratory disease (ARD) in military personnel. However, these two vaccines were used until the 1990s [4]. They have been lost when the production ceased. A new question that could be asked is whether the candidate AdV4 and AdV7 vaccines would protect against AdV14 [4]. In light of not having an approved adenovirus vaccine, antiviral treatment remains the other option. Unfortunately, no antiviral agents have yet been approved for the treatment of adenovirus infections. Therefore, development of new antiviral agents is urgently needed for the treatment of patients with adenovirus, particularly serotype 14 infection.

Hartline et al. reported the inhibitory activities of ether lipid-ester prodrugs of acyclic nucleoside phosphonates against five adenovirus serotypes [10]. The compounds were cidofovir (CDV) analogues and analogues of (S)-HPMPA, hexadecyloxypropyl-(S)-HPMPA (HDP-HPMPA), octadecyloxyethyl-(S)-HPMPA (ODE-HPMPA), hexadecyloxypropyl-CDV (HDP-CDV), octadecyloxyethyl-CDV (ODE-CDV), oleyloxyethyl-CDV (OLE-CDV), and oleyloxypropyl-CDV (OLP-CDV), tetradecyloxypropyl-CDV (TDP-CDV) and eicosyloxypropyl-CDV (ECP-CDV), new analogues of CDV with an alkoxyalkyl structure; and actyl-CDV (O-CDV), dodecyl-CDV (DD-CDV), eicosyl-CDV (EC-CDV), docosyl-CDV (DC-CDV), and tetracocyl-CDV (TC-CDV), alkyl esters with no linker moiety. Many of the alkoxyalkyl compounds had excellent *in vitro* activities with the high antiviral selectivity. One of them, ODE-HPMPA was also expected to be active when given orally and was considered a good candidate for further study. In the current report, we evaluated additional ODE-nucleoside analogues, especially against a clinical isolate of AdV14, and found to be effective *in vitro*.

## Materials and methods

### Cells

A549 cells, a human lung carcinoma cell line, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were routinely grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific Inc., Logan, UT). For antiviral assay, the serum was reduced to 2% and gentamicin was added to the medium at a final concentration of 50 µg/ml.

### Viruses

Human AdV14 (VR-15) was provided by the Centers for Disease Control Control (CDC, Atlanta, GA). The original isolate was obtained from the throat washing of recruit with acute respiratory illness in Netherlands, in 1955. AdV1 (strain 65089), which was isolated from the tracheal washing of a pediatric patient, was provided from M.F. Smaron (Department of Medicine, University of Chicago, Chicago, IL). AdV2 (strain Miller), AdV5 (strain Adenoid 75, VR-5), and, AdV7, (strain Gomen), AdV7 (strain 97-185), AdV7 (strain Ferrell), AdV11 (strain Slobitski), AdV15 (strain B 1869), were obtained from ATCC. AdV48 (strain 10683), AdV48 (strain 28713), AdV48 (strain 7862), AdV48 (strain 9081), and AdV48 (strain 10884), were from ARUP Laboratories (University of Utah, Salt Lake City, UT). All the strains were propagated and titrated in A549 cells.

### Test compounds

Octadecyloxypropyl esters of 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)-adenine (ODE-HPMPA) (MW: 599.38), octadecyloxypropyl esters of 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)-cytosine (ODE-HPMPC) (MW: 597.7), octadecyloxypropyl esters of 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)-guanine (ODE-HPMPG) (MW: 615.74), and octadecyloxypropyl esters of 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)-2,6-diaminopurine (ODE-HPMP-DAP) (MW: 614.76) were obtained from University of California San Diego (La Jolla, CA). 2',3'-Dideoxycytidine (MW: 211.22) [11, 12] was purchased from Sigma-Aldrich (St. Louis, MO). All the ODE-nucleoside analogues were dissolved in phosphate buffered saline (PBS) for *in vitro* experiments. 2',3'-Dideoxycytidine was dissolved in cell culture medium.

**Cytopathic effect (CPE) inhibition assay**

A modified protocol of Barnard et al. [13] was used for the *in vitro* evaluation of antiviral efficacy of the inhibitors of adenovirus replication. A549 cells were seeded onto 96-well tissue culture plates (Corning Incorporated Costar, NY). Each ODE-nucleoside analog and virus were added in equal volumes to near-confluent cell monolayers in 96-well tissue culture plates the next day. The multiplicity of infection (MOI) used was approximately 0.001 in order to produce complete virus cytopathic effects (CPE) in untreated cell monolayers within 3–4 days. The plates were incubated at 37°C until the cells in the virus control wells showed complete viral CPE as observed by light microscopy. Each concentration of drug was assayed for inhibition of viral CPE in triplicate and for cytotoxicity in duplicate uninfected wells. Six wells per plate were set aside as uninfected, untreated cell controls and six wells per plate received virus only and represented controls for virus replication. 2',3'-

dideoxycytidine was tested as the positive control drug for each set of compounds tested.

Morphological changes resulting from cytotoxicity of each ODE-nucleoside analog or virus CPE were graded on a scale of 0–5, with 5 defined as the appearance of complete cytotoxicity or CPE involving the entire monolayer as observed by light microscopy. The values obtained were then converted to percentages of untreated, uninfected controls. The 50% cell cytotoxic concentrations ($CC_{50}$) and 50% virus inhibitory concentrations ($IC_{50}$), representing the putative concentration at which 50% of the monolayers would show compound cytotoxicity or virus CPE, respectively, were estimated by regression analysis. A selectivity index (SI) value was calculated using the formula as $SI = CC_{50}/IC_{50}$. The activity in the CPE assay was then verified spectrophotometrically by neutral red (NR) uptake assay on the same plate.

**Neutral red (NR) uptake assay for determination of antiviral efficacy and cytotoxicity of the ODE-nucleoside analogues**

This assay was done for each CPE inhibition test plate described above to verify the inhibitory activity and the cytotoxicity detected by visual observation. In our experience, the usual correlation between visual and neutral red (NR) uptake assays in our hands has been greater than 95%. The neutral red (NR) uptake assay was performed using a modified method of Cavanaugh et al. [14] as described by Barnard et al. [15]. Briefly, medium was removed from each well of a plate, 0.034% neutral red (NR) was added to each well of the plate, and the plate was incubated for 2 h at 37°C in the dark. The neutral red (NR) solution was removed from the wells, the wells were rinsed and any remaining dye was extracted using Sörenson's citrate buffered

ethanol (pH 4.2). Absorbances at 540 nm/405 nm were read with a microplate reader (Opsys MR™, Dynex Technologies, Chantilly, VA). Absorbance values were expressed as percentages of untreated controls and $IC_{50}$, $CC_{50}$, and SI values were calculated as described above.

**Virus yield reduction assay**

Virus yield reduction (VYR) assay was used to confirm the results of the CPE inhibition/NR uptake assays. Infectious virus yield from the CPE inhibition assay were determined on the supernatant from the test well as previously described [15]. After the CPE was scored as described above, each plate was frozen at −80°C and then thawed. Sample wells at the concentrations of each ODE-nucleoside analog were pooled and titrated in A549 cells for infectious virus by CPE assay as previously described by Barnard et al. [13]. A 90% reduction in virus yield ($IC_{90}$) was then calculated by linear regression analysis. This value represented a one-$\log_{10}$ inhibition in titer when compared to untreated virus controls.

**Time-of-addition assay**

To determine the target step of the ODE-nucleoside analogues in the adenovirus life cycle, the time-of-addition assay was performed according to the method previously described [16, 17].

**Results**

**Effects of various concentrations of ODE-nucleoside analogues on cytotoxicity**

We first examined the effects of various concentrations of ODE-nucleoside analogues on the cytotoxicity to A549 cells. This was calculated as the concentration of the ODE-nucleoside analogues capable of reducing neutral red dye uptake by 50% compared to untreated cells. The ODE-nucleoside analogues were cytotoxic to A549 cells at high concentrations ranging from 420 ± 310 nM to 9600 ± 1100 nM after 3 days of incubation (Table 1).

**Effect of the ODE-nucleoside analogues on AdV14 infection *in vitro***

We next examined the effects of different concentrations of the four ODE-nucleoside analogues on the AdV14 infection in A549 cells. All of the four ODE-nucleoside analogues also inhibited the adenovirus 14 isolate with SI values ranging from 32 ± 1.0 to 877 ± 287 as determined by neutral red uptake assay (Table 2). ODE-HPMP-DAP was the most potent among them with an $IC_{50}$ of 1.7 ± 0.19 nM determined by visual assay and, an $IC_{50}$ of 1.4 ± 0.05 nM determined by neutral red (NR) uptake assay (Table 2).

The activity of four ODE-nucleoside analogues was confirmed in a virus yield reduction assay. ODE-HPMP-DAP reduced virus yields of the AdV14 by 90% at 4.1 ± 2.5 nM in A549 cells, which correlated well with the potent activity detected by neutral red (NR) uptake assay. The other ODE-nucleoside analogues, ODE-HPMPC and ODE-HPMPA as well as ODE-HPMPG, also blocked AdV14 replication, reducing virus yields by 90%, with $IC_{90}$ values ranging from 6.5 ± 1.1 to 20 ± 4.2 nM (Table 2).

**Effect of the ODE-nucleoside analogues on other adenovirus infections *in vitro***

We also examined whether the ODE-nucleoside analogues inhibited adenovirus serotype 1, 5 and 7. A549 cells were seeded and then infected with AdV1, AdV5, or AdV7, respectively. ODE-nucleoside analogues were active against all of these adenovirus strains with $IC_{50}$ values ranging from 0.3 ± 0.1 nM to 61 ± 13 nM for the visual assay, or from 0.2 ± 0.1 nM to 42 ± 17 nM for the neutral red uptake assay (Table 3). These results were also confirmed by the virus yield reduction assay, with $IC_{90}$ values ranging from 1.6 ± 0.85 nM to 120 ± 200 nM (Table 3). Thus, the ODE-nucleoside analogues inhibited the adenovirus infections *in vitro*.

To further examine the effects of ODE-HPMPA on infection with additional adenovirus serotypes such as AdV2 (strain Miller), AdV7 (strains 97-185 and Ferrell), AdV11, AdV15, AdV48 (strains of 10683, 28713, 7862, 9081, 10884), the neutral red uptake assay was performed as described above. The inhibitory effects of the ODE-HPMPA compound were similar, irrespective of whether A549 cells have been infected with some other adenovirus serotypes (data not shown). Similar results were also obtained when A549 cells had been treated with ODE-HPMPC or ODE-HPMPG or ODE-HPMP-DAP and then, infected with some other adenovirus serotypes (data not shown).

**Effect of time-of-addition assay on a single cycle of virus replication**

A549 cells were infected with adenovirus type 14. Then, the ODE-nucleoside analogues (ODE-HPMPA, ODE-HPMPC, ODE-HPMPG, ODE-HPMP-DAP) at 100 nM, which is much higher than the $IC_{50}$, were added to the cells at various time points after infection. The ODE-nucleoside analogues were shown to inhibit the early phase (Table 4) after infection, but not the late phase (8, 12 or 24 h after infection), of adenovirus life cycle. Our data suggest that the ODE-nucleoside analogues act on an

early step of adenovirus infection, presumably on DNA synthesis [18] and since these are all nucleoside analogs.

## Discussion

Acyclic nucleoside phosphonates (ANPs) represent a key class of antiviral agents [19]. Various nucleoside derivatives of 3-hydroxy-2-phosphonomethoxypropyl (HPMP) were shown to have the antiviral activities [20]. However, these types of compounds were also shown to have poor bioavailability and renal toxicity [21, 22]. To overcome these drawbacks, octadecyloxyethyl (ODE) esters were added to HPMP compounds to increase bioavailability and antiviral efficacy [23]. In this report, we tested the antiviral activities of ODE-HPMPA, ODE-HPMPC, ODE-HPMPG and ODE-HPMP-DAP against AdV14 and other adenovirus serotypes in A549 cells. All the ODE-nucleoside analogues demonstrated significant antiviral activities in neutral red uptake assay and virus yield reduction assay *in vitro*.

9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl) adenine [(*S*)-HPMPA] is an acyclic nucleoside phosphonate which Holý and his coworkers first reported in 1986 [20, 24]. (*S*)-HPMPA was one of a growing and important class of antiviral compounds which now includes cidofovir, adefovir [9-(2-phosphonomethoxyethyl) adenine], and tenofovir [9-(2-phosphonomethoxypropyl) adenine], which are used for the treatment of virus infections [25]. (*S*)-HPMPA is a broad-spectrum antiviral which was shown to inhibit the replication of a wide variety of double-stranded DNA viruses, including cytomegalovirus [26-28], orthopoxviruses [27, 29], herpesviruses [26, 29], and adenoviruses [20, 24, 25]. (*S*)-HPMPA was also reported to be active *in vitro* against HBV replication in HB611 cells [30] and 2.2.15 cells. Morrey et al. demonstrated that

oral treatment of HBV transgenic mice with HDP-(S)-HPMPA, 15M-HDP-(S)-HPMPA, and ODE-(S)-HPMPA for 14 days reduced liver HBV DNA level by roughly 1.5 log units [31]. It has been reported that alkoxyalkyl esters of (S)-9-(3-hydroxy-2-phosphonomethoxypropyl) adenine are also potent inhibitors of hepatitis C virus replication in genotype 1A, 1B, and 2A replicons [32] and HIV-1 replication *in vitro* [33, 34]. Hartline et al. tested the inhibitory effect of ODE-HPMPA on AdV3, AdV5, AdV7 AdV8 and AdV31 in human foreskin fibroblast (HFF) cells by a plaque-reduction assay [10]. ODE-HPMPA was shown to have an $IC_{50}$ value of 40 ± 40 nM against AdV5 and, an $IC_{50}$ value of 60 ± 80 nM against AdV7. We determined that the ODE-HPMPA had an $IC_{50}$ of 21 ± 2.1 nM against AdV5 and, an $IC_{50}$ of 2.6 ± 0.78 nM against AdV7 in A549 cells. However, to our knowledge, this is the first report that the ODE-nucleoside analogues inhibited AdV14 infection *in vitro*.

Mul et al. studied the mechanism of inhibition using a reconstituted *in vitro* DNA replication system and found that (S)-HPMPA blocked adenovirus DNA polymerase at the level of chain elongation [18], suggesting that the adenovirus DNA polymerase is the prime target for the drug. Our findings are consistent with their report: the ODE-nucleoside analogues were shown to inhibit the early phase (0, 2, or 4 h) after infection, but not the late phase (8, 12 or 24 h after infection), of adenovirus life cycle, suggesting that the ODE-nucleoside analogues would act on an early step of adenovirus infection. Taken together the ODE-nucleoside analogues offer low micromolar antiviral activity against adenovirus infections and, are the leading compounds, which can be further modified. Our data provide important insight into a series of compounds that might be advantageous in treating adenovirus infections.

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## Disclosure statement

The authors declare no competing interests.

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